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REVIEW

Formation and regulation of *Yersinia* biofilms

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ABSTRACT

Flea-borne transmission is a recent evolutionary adaptation that distinguishes the deadly *Yersinia pestis* from its progenitor *Y. pseudotuberculosis*, a mild pathogen transmitted via the food-borne route. *Y. pestis* synthesizes biofilms in the flea gut, which is important for flea-borne transmission. *Yersinia* biofilms are bacterial colonies surrounded by extracellular matrix primarily containing a homopolymer of *N*-acetyl-*D*-glucosamine that are synthesized by a set of specific enzymes. *Yersinia* biofilm production is tightly regulated at both transcriptional and post-transcriptional levels. All the known structural genes responsible for biofilm production are harbored in both *Y. pseudotuberculosis* and *Y. pestis*, but *Y. pestis* has evolved changes in the regulation of biofilm development, thereby acquiring efficient arthropod-borne transmission.

KEYWORDS Yersinia pestis, *Y. pseudotuberculosis*, biofilm, flea-borne transmission

INTRODUCTION

Yersinia pestis is a clone diverged recently (from the evolutionary point of view) from *Y. pseudotuberculosis* (Achtman et al., 1999) and the two pathogens share a very high genomic homology of genomic structure and content (Chain et al., 2004). *Y. pseudotuberculosis* generally causes mild enteric diseases that are transmitted via the food-borne route. *Y. pestis* is highly virulent to mammalians and causes systemic and fatal infections, e.g., bubonic, septicemic, and pneumonic plague (Perry and Fetherston, 1997). Plague infections are mostly zoonotic with humans being the accidental hosts. Outbreaks of plague occur throughout human history, especially including three major pandemics that claimed hundreds of thousands of lives. Transmission of *Y. pestis* relies primarily on the bite of flea vectors, although infection can occur through direct contact or inhalation. *Y. pestis* forms biofilms attached to the flea gut and can starve fleas by blocking their proventriculi, which stimulates the insects to bite repeatedly and thereby infect new mammalian hosts (Darby, 2008). *Yesinia* biofilms are a population of bacterial colonies that are surrounded by extracellular matrix containing primarily a homopolymer of *N*-acetyl-*D*-glucosamine (Hinnebusch and Erickson, 2008). The complex cascades of transcriptional and post-transcriptional regulation involved in *Yersinia* biofilm formation, as well as its contribution to the evolution of flea-borne *Y. pestis* transmission, are discussed in this review.

MICROBIAL BIOFILMS

A biofilm (Fig. 1) is a community of microorganisms embedded within a self-produced matrix of extracellular polymeric substances that are a polymeric conglomeration of polysaccharides, proteins, nucleic acids, and lipids (Flemming and Wingender, 2010). Being physiologically distinct from their planktonic counterparts in liquid medium, cells in a biofilm are protected from antibacterial molecules, bacteriophages, antibodies, and phagocytes, and other antibacterial mechanisms. Thus, biofilm formation represents a mode of protection for the growth that allows microbial cells to survive and propagate in various adverse environments, including natural, industrial or hospital settings, and infectious sites (Donlan and Costerton, 2002; Davies, 2003; Hall-Stoodley et al., 2004; Fux et al., 2005). Therefore, biofilm formation contributes to not only the environmental survival and transmission of microorganisms, but also to chronic infection by pathogens resistant to both host immunity and antibiotic therapy (Donlan and Costerton, 2002; Davies, 2003; Hall-Stoodley et al., 2004; Fux et al., 2005).

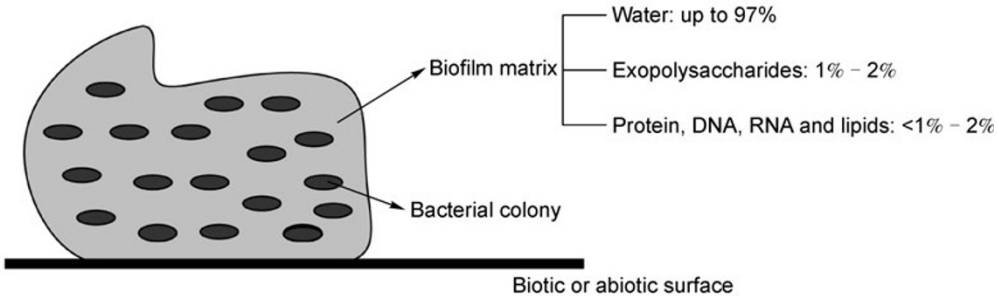

Figure 1. A microbial biofilm

BIOFILM FORMATION AND PLAGUE TRANSMISSION

Compared with *Y. pseudotuberculosis*, *Y. pestis* utilizes a radically different mechanism of transmission in rodent reservoirs, relying primarily on the bite of flea vectors. The development of heavy bacteremia in rodents is crucial to reliably infect fleas, which transmit the disease by biting a new host animal (Lorange et al., 2005). Infected fleas often develop "blockage" in the proventriculi of fleas (Darby, 2008; Hinnebusch and Erickson, 2008). *Y. pestis* synthesizes biofilms to attach onto the surface of the proventricular spines, and the heavy bacterial proliferation in the biofilms promotes the blockage of the gut of fleas (see reference (Darby, 2008) for a schematic representation of a blocked flea). Blockage of fleas inhibits feeding, and makes them feel hungry and repeatedly attempt to feed, during which the ingested blood will be regurgitated back into the bite sites, causing *Y. pestis* infection of the new hosts.

The relative long time (about two weeks) for blockage development is not sufficient to explain the rapid spread that typifies plague epidemics (Eisen et al., 2006; Eisen and Gage, 2009). Infected fleas are immediately infectious and transmit the microorganisms efficiently for at least 4 days post infection, and the mode of "early-phase transmission" by unblocked fleas has been proposed accordingly (Eisen et al., 2006; Eisen et al., 2008). During testing of an early-phase transmission model, defects in *Y. pestis* biofilm formation do not prevent flea-borne transmission, whereas biofilm over-production inhibits efficient early-phase transmission (Vetter et al., 2010). Unlike the traditional blockage-dependent plague transmission model, early-phase transmission can occur when a flea takes its first blood meal after initial infection by feeding on a bacteremic host, which may explain the rapid spread of disease from fleas to mammalian hosts during an epizootic (Eisen et al., 2006; Eisen et al., 2008).

CAENORHABDITIS ELEGANS AS A MODEL FOR BIOFILM STUDY

The lawns of biofilm-negative *Escherichia coli* OP50, a uracil auxotroph whose growth is limited on NGM (Nematode Growth Medium) agar plates, are frequently used as the standard food for *C. elegans*. When the larvae or adults of *C. elegans* grow on lawns of *Y. pestis*, the bacterium creates a biofilm, which primarily covers the head of the nematode by blanketing the mouth, thereby preventing the nematode from feeding. This has been developed as a model for *Yersinia* biofilm research (Darby et al., 2002; Joshua et al., 2003; Tan and Darby, 2004). The matrix of the *Yersinia* biofilm attaches directly to the nematode cuticle (Fig. 2). As the animal moves through the bacterial lawn, the matrix becomes progressively thicker as additional material accumulates on top of the initial layer (Darby et al., 2002; Joshua et al., 2003; Tan and Darby, 2004). The covering of the nematode mouth by *Yersinia* biofilm blocks nematode feeding, thereby inhibiting its growth and development, which provides a quantitative assay for biofilm production (Darby et al., 2005). When the nematode eggs were incubated on bacterial lawns and the developmental stages were scored three days later, almost all of the scored eggs developed to the L4 (4th stage larvae) or adult when OP50 was used as food, whereas a considerably smaller percentage (about 30% for *Y. pestis* microtus strain 201, unpublished data) of those exposed to *Y. pestis* grew to L4 or adulthood (Li et al., 2008). This scheme can be used to test the biofilm forming ability of different wild-type *Y. pseudotuberculosis* and *Y. pestis* strains, as well as genetically modified strains (Darby et al., 2005; Erickson et al., 2006a; Tan and Darby, 2006). The *C. elegans* biofilm system may prove useful as a general model for bacterial biofilm formation on a living surface, which could not only be used to characterize the bacterial determinants of biofilm formation, but also provide insights into the means by which bacterial pathogens adhere to host tissues in biofilm-mediated infectious diseases (Drace and Darby, 2008).

STRUCTURAL DETERMINANTS OF BIOFILM PRODUCTION

A set of factors have been linked to *Yersinia* biofilm production (Table 1). The three separate operons *hmsHFRS*, *hmsT*, and *hmsP* are involved in the Pgm^+ phenotype which is

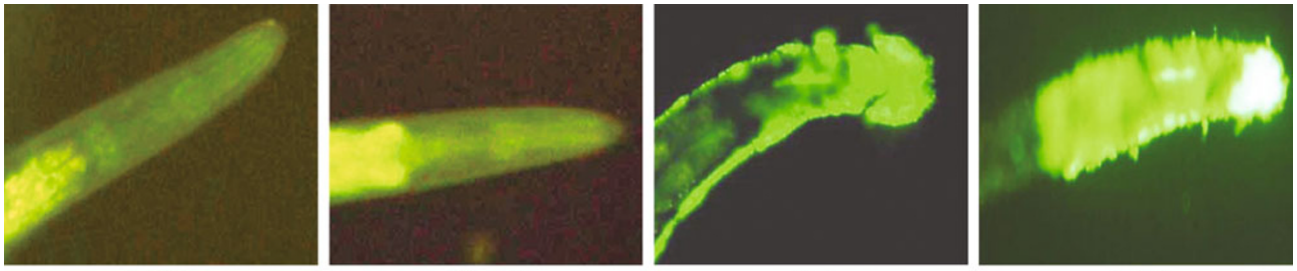

Figure 2. *Yersinia* biofilms on nematode. The tested bacteria included *E. coli* OP50, the wild-type (WT), and the *hmsS* (*ΔhmsS*) and *phoP* (*ΔphoP*) null mutants of *Y. pestis* microtus strain 201 (Li et al., 2008). Bacterial strains were transformed with the plasmid pBC-GFP expressing green fluorescent protein (Matthysse et al., 1996). When the adult or L4 nematodes were placed on the bacterial lawns, *Yersinia* biofilms adhered to the surface of nematode, primarily on the head to cover the mouth. *ΔphoP* produced more extensive and denser biofilms than WT, and yet no biofilm was detectable in *ΔhmsS* (negative control) and OP50 (blank control).

Table 1 Functions involved in *Yesinia* biofilm formation

Gene ID	Gene name	Function	References
		Structural determinants	
YPO1951-1954	*hmsHFRS*	Biosynthesis of biofilm matrix	Lorange et al., 2005
YPO3243	*gmhA*	Biosynthesis of heptose	Darby et al., 2005
YPO3577	*yrbH*	Biosynthesis of Kdo	Tan and Darby, 2006
YPO0055-0053	*waaAE-coaD*	Transfer of Kdo to LPS	Tan and Darby, 2006
		Post-transcriptional regulation	
YPO0425	*hmsT*	Biosynthesis of c-di-GMP biosynthesis	Kirillina et al., 2004; Simm et al., 2005
YPO3996	*hmsP*	Degradation of c-di-GMP degradation	Kirillina et al., 2004; Bobrov et al., 2005
YPO0929	*speA*	Biosynthesis of polyamines	Patel et al., 2006; Wortham et al., 2010
YPO1201	*speC*		
YPO2632	*nghA*	Degradation of biofilm matrix	Erickson et al., 2008
		Transcriptional regulation	
YPO2449	*rcsA*		
YPO1217	*rcsC*	Rcs phosphorelay system	Sun et al., 2008
YPO1219-1218	*rcsDB*		
YPO1633-1634	*phoPQ*	Two component system PhoP/Q	Sun et al., 2009

characterized by the formation of greenish-brown and red pigmented colonies on hemin and Congo red plates, respectively (Hinnebusch and Erickson, 2008). The operon *hmsHFRS* is responsible for the synthesis of poly β-1,6-nacetyl-D-glucosamine exopolysaccharides, a key component of the biofilm matrix in many biofilm-forming bacteria, and is required for biofilm formation and for flea blockage of *Y. pestis* (Hinnebusch and Erickson, 2008). HmsH and HmsF are outer membrane proteins, whereas the other four Hms proteins are located in the inner membrane (Abu Khweek et al., 2010). HmsH has a β-barrel structure with a large periplasmic domain. HmsF possesses polysaccharide deacetylase and glycosyl hydrolase (COG1649) domains whereas HmsR is a putative glycosyl transferase, and HmsS has no recognized domains (Forman et al., 2006; Abu Khweek et al., 2010). Some but not all of the substitutions within the periplasmic domain of HmsH and HmsS are critical for their protein function. Substitutions within the deacetylase domain of HmsF and within the glycosyltransferase domain of HmsR abolish biofilm formation. Surprisingly, substitution of highly conserved residues within COG1649 does not affect HmsF function. Multiple interactions have been detected for the Hms inner membrane proteins, which indicate that the synthesis and regulation of biofilm exopolysaccharides occurs in the cytoplasm through a proposed Hms enzymatic complex (Bobrov et al., 2008).

GmhA is a phosphoheptose isomerase required for heptose synthesis, a conserved component of lipopolysaccharide (LPS) and lipooligosaccharide, and *gmhA* mutants have severely hampered biofilm formation inadequate for flea blockage (Darby et al., 2005). YrbH is an arabinose 5-phosphate isomerase that catalyzes the conversion of ribulose 5-phosphate into arabinose 5-phosphate, the first committed step in the 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) biosynthesis pathway, whereas WaaA is a transferase catalyzing Kdo glycosylation of lipid A (Tan and Darby, 2005). The *yrbH* and *waaA* mutants of *Y. pestis* have reduced growth rates and the synthesized LPS lacks Kdo (Tan and Darby, 2005). The *yrbH* mutant absolutely failed to form biofilms, whereas the *waaA* mutant produces defective but detectable biofilms (Tan and Darby, 2006).

TRANSCRIPTIONAL REGULATION OF BIOFILM FORMATION

The Rcs phosphorelay system is composed of RcsC, RcsD, RcsB, and RcsA (Majdalani and Gottesman, 2005). The sensor kinases RcsC and RcsD transfer phosphate to RcsB; activated by the phosphorylation, the DNA-binding regulator RcsB regulates the transcription of some targets independently, whereas other targets require the accessory protein RcsA (Majdalani and Gottesman, 2005). The Rcs system negatively regulates *Yersinia* biofilm production in both nematode and flea models (Sun et al., 2008). The RcsAB consensus-like sequences are located within the promoter regions of both *hmsHFRS* and *hmsT*, indicating that they might serve as the direct targets of RcsAB.

The PhoP/PhoQ two-component system is composed of the inner membrane sensor PhoQ and cytoplasmic responsive regulator PhoP. *Y. pestis* appears to use host macrophages as a protective niche during the early stages of infection (Lukaszewski et al., 2005). PhoP controls the ability of *Y. pestis* to survive in macrophages, most likely through stimulating the expression of a set of factors important for survival against macrophage-killing mechanisms, especially MgtCB and Ugd, which are required for the bacterial replication in macrophages (Grabenstein et al., 2006). PhoP represses the formation of *Yersinia* biofilms on both nematode and plastic surfaces (Sun et al., 2009). Deletion of *phoP* in *Y. pseudotuberculosis* induced the production of robust biofilms, whereas a *phoP* null mutant of *Y. pestis* produced more extensive biofilms than the wild type. Expression of HmsT, a diguanylate cyclase that positively regulates biofilms, is diminished in *Y. pseudotuberculosis* strains with functional PhoP (Sun et al., 2009). HmsT is absent or barely detectable in the wild-type *phoP* present in *Y. pseudotuberculosis*, but it is highly expressed when *phoP* mutates or is deleted (Sun et al., 2009). PhoP consensus-like sequences are located within the promoter region of *hmsT*, indicating this gene might serve as the direct target of PhoP.

POST-TRANSCRIPTIONAL REGULATION OF BIOFILM FORMATION

The signaling molecule 3′,5′-cyclic diguanylic acid (c-di-GMP) is a positive allosteric activator of enzymes used by bacteria to produce biofilm matrices (Hengge, 2009; Schirmer and Jenal, 2009). Through interaction with different receptors (e.g., PilZ-containing proteins), c-di-GMP negatively modulates cell motility and pathogenicity, but stimulates biofilm-associated pathways (Hengge, 2009; Schirmer and Jenal, 2009). c-di-GMP is produced from GTP by diguanylate cyclases, and is degraded by phosphodiesterases. The GGDEF domain is generally the catalytically active part of diguanylate cyclases, whereas the EAL and HD-GYP domains are two alternative and structurally unrelated domains with phosphodiesterase activity (Hengge, 2009; Schirmer and Jenal, 2009). The cellular concentrations of c-di-GMP are tightly determined by the above domains of diguanylate cyclases and phosphodiesterases, and these two enzymes are typically controlled by internal or external signals that are sensed directly or indirectly by accessory domains (Hengge, 2009; Schirmer and Jenal, 2009).

HmsT is a GGDEF domain-containing protein that synthesizes c-di-GMP and stimulates *Y. pestis* biofilm formation, and the mutation of *hmsT* results in poor biofilm formation (Kirillina et al., 2004; Simm et al., 2005). HmsP, an EAL-domain protein, has the c-di-GMP-specific phosphodiesterase activity and is involved in the degradation of c-di-GMP, and therefore has a negative effect on biofilm formation (Kirillina et al., 2004; Bobrov et al., 2005). Alanine substitutions for each of the GGEE residues of HmsT, as well as the E506 and L508 residues of HmsP, cause a loss of function (Kirillina et al., 2004; Bobrov et al., 2005; Simm et al., 2005). The virulence of *hmsT* mutants incapable of c-di-GMP synthesis is unaffected in plague mouse models; conversely, *hmsP* mutants unable to degrade c-di-GMP have defective virulence via the subcutaneous route of infection due to poly-beta-1,6-N-acetylglucosamine overproduction, suggesting that c-di-GMP signaling is not only dispensable but deleterious for *Y. pestis* virulence (Bobrov et al., 2011).

The genes *speA* (arginine decarboxylase) and *speC* (ornithine decarboxylase) are involved in polyamine biosynthesis. Mutation of *speA* or *speC* causes a loss of *in vitro* biofilm formation in *Y. pestis* (Patel et al., 2006). Through a still unclear post-transcriptional regulation mechanism, polyamines are necessary for maintaining the levels of key Hms proteins such as HmsR, HmsS, and HmsT (Wortham et al., 2010). Remarkably, polyamines play a role in bubonic plague (Wortham et al., 2010).

The *Y. pseudotuberculosis* NghA is a glycosyl hydrolase that cleaves β-linked N-acetylglucosamine residues through hydrolysis of the growing polymers at any point during their initial assembly in the cytoplasm. It transports through the periplasm, or extracellular anchoring to the cell envelope,

thereby degrading the biofilm matrix and reducing biofilm formation (Erickson et al., 2008).

EVOLUTION OF FLEA-BORNE TRANSMISSION

All of the known structural genes required for biofilm formation are harbored in *Y. pseudotuberculosis*, which has 99% homology with their *Y. pestis* counterparts, but typical *Y. pseudotuberculosis* has a Pgm-phenotype and cannot synthesize adhesive biofilms on nematodes (Erickson et al., 2006b). The negative effect on Hms-dependent biofilm matrix production by multiple factors, including Rcs, PhoP, and NghA, produces a tight biofilm-phenotype typical of *Y. pseudotuberculosis*. However, a very small number of *Y. pseudotuberculosis* strains have the biofilm+ phenotype in the nematode model; among them is *Y. pseudotuberculosis* YPIII, a widely used laboratory strain that makes strong biofilms on nematodes due to *phoP* inactivation (Sun et al., 2009). The inactivation of one or more biofilm-inhibitory factors (e.g., PhoP/Q regulatory system in YPIII) would disturb the tight inhibition of biofilm production, and induce a nematode-based biofilm+ phenotype in a few *Y. pseudotuberculosis* strains (Fig. 3) (Sun et al., 2009).

None of the tested *Y. pseudotuberculosis* strains has the ability to form adhesive biofilms in fleas, which might be due to the absence of the Yersinia murine toxin (Ymt). The *ymt* gene is located on the pMT1 plasmid, which is acquired by *Y. pestis* (Parkhill et al., 2001). Ymt has phospholipase D activity that likely protects *Y. pestis* from unknown cytotoxic effects in the flea gut environment; hence, it is required for bacterial survival in fleas (Hinnebusch et al., 2002). The acquisition of Ymt by *Y. pestis* provides a prerequisite for bacterial survival in the flea gut.

Both RcsA and NghA are functional in *Y. pseudotuberculosis*, but their structural genes are inactivated in *Y. pestis* (Erickson et al., 2008; Sun et al., 2008). Expression of functional RcsA or NghA in *Y. pestis* strongly represses biofilm formation and essentially abolishes flea blockage (Erickson et al., 2008). NghA and RcsA may represent an anti-transmission factor for biofilm formation and flea-borne transmission. During its speciation from *Y. pseudotuberculosis*, *Y. pestis* undergoes the positive selection of genetic inactivation (rather than neutral mutation) of at least two inhibitory functions (Rcs and NghA) on biofilm development, which provides it with a strong ability to synthesize adhesive biofilms and becomes an incremental step in the evolution of vector-borne transmission (Fig. 3). These evolutionary events enable *Y. pestis* to survive in fleas and synthesize adhesive biofilms in flea proventriculi, resulting in efficient arthropod-borne transmission.

CONCLUDING REMARKS

The causative agent of plague *Y. pestis* cycles permanently among many species of wild rodents and fleas in enzootic foci, making the pathology and epidemiology of plague quite complex. Flea-borne transmission is a recent evolutionary adaptation that distinguishes *Y. pestis* from its progenitor *Y. pseudotuberculosis* that is a mild pathogen transmitted via the food-borne route. In contrast to *Y. pseudotuberculosis*, *Y. pestis* is able to grow and synthesize the attached biofilms in the proventriculus of flea vectors, which promotes the transmission. This mode of transmission has not been described for any pathogen except *Y. pestis*. *Yersinia* biofilms are a population of bacterial colonies surrounded by exopolysaccharide matrix that the microbes themselves produce. The production of exopolysaccharide matrix is tightly regulated at both transcriptional and post-transcriptional levels. The positive selection of genetic changes in the relevant regulation circuit makes *Y. pestis* evolve a robust ability to develop biofilms on both biotic and abiotic surfaces, although all the known structural genes responsible for biofilm production are harbored in both *Y. pseudotuberculosis* and *Y. pestis*. In addition, the

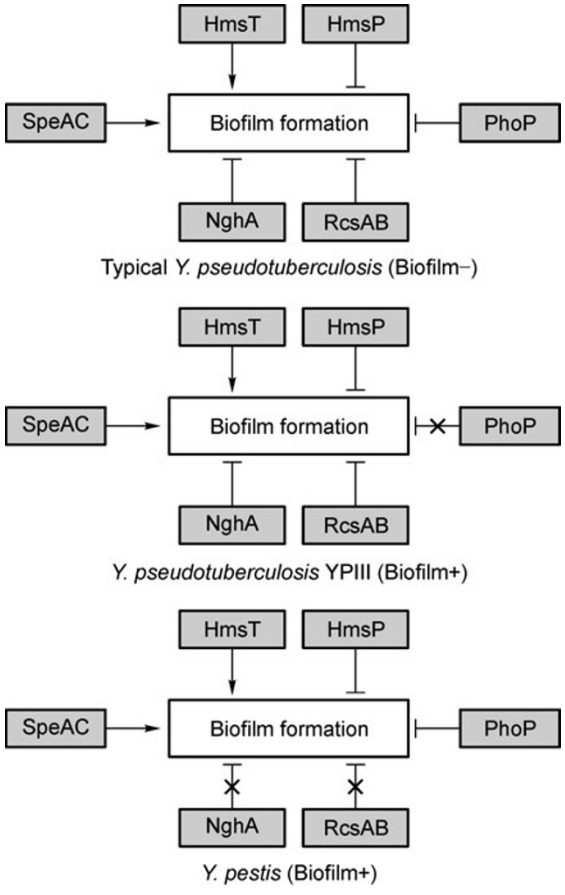

Figure 3. Regulation of *Yersinia* biofilm formation. Lines with arrows indicate positive regulation, whereas those with a bar denote negative regulation. A cross indicates elimination of the corresponding regulation pathway.

acquisition of Ymt provides a prerequisite for *Y. pestis* to survive in the flea gut. Apparently, these discrete genetic changes contribute to the evolution of arthropod-borne transmission. Prevention and control of plague involve the control of fleas and rodents, isolation of human victims, treatment or prophylaxis with antibiotics, and vaccination of persons at high risk. The pathways of formation and regulation of *Yersinia* biofilms may uncover novel targets for the prevention or treatment of this deadly disease.

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